HYDROLYSIS OF ANIMAL PROTEIN MEALS FOR IMPROVED UTILITY IN NON-FEED APPLICATIONS

R. A. Garcia, D. J. Pyle, G. J. Piazza, Z. Wen

ABSTRACT. *Rendered proteins are well suited for animal nutrition applications, but due to their insolubility, inhomogeneity, and the presence of non-protein substances, they are difficult to utilize in other applications. In an attempt to overcome these obstacles to utilization, three types of rendered proteins [meat and bone meal (MBM), feather meal (FM), and blood meal (BM)] were partially defatted and then hydrolyzed to varying extents using calcium hydroxide or one of three enzymatic treatments, in 4- or 6-L batches. After centrifugation, filtration, and spray drying, these hydrolysates were analyzed for changes in physical and chemical properties that relate to their potential utility. In all cases, the proportion of organic matter solubilized increased along with hydrolysis duration, although the molar mass distribution of the hydrolysis product only had a weak dependence on hydrolysis duration; the soluble material consisted of very small peptides at all time points. Alkali-hydrolysis was not effective in yielding a product low in ash; although the insoluble ash in MBM and FM appears not to have been carried over into the product, it was replaced by significant amounts of calcium salts; corresponding enzymatically-hydrolyzed batches contained approximately 40% less ash. Alkali-hydrolysis in particular had effects on the amino acid composition of the products, destroying some amino acids and creating others, including the cross-linked amino acids lysinoalanine and lanthionine; enzymatic hydrolysis effects on amino acid composition were different in type and generally lesser in magnitude. It is concluded that hydrolysis is a promising treatment for increasing the non-feed utility of rendered animal proteins.*

Keywords. *Rendered protein, Meat and bone meal, Hydrolysis, Solubilization, Molar mass distribution.*

In North America, the low-value tissues from farm animal carcasses are typically used as high-protein components in compound animal feed. For this application it is critical that these materials be stable without refrigeration, free of pathogens, and highly digestible (Meeker, 2006). These characteristics are achieved by processing at a rendering plant. Bones and offal are rendered at high temperature yielding meat and bone meal (MBM) as one product; feathers are cooked in pressure vessels yielding "hydrolyzed" feather meal (FM) (Nissen, 1995); blood is coagulated and dried yielding blood meal (BM) (Fernando, 1992). While these meals are well suited to animal nutrition, due to their poor solubility, inhomogeneity, and intermingling with non-protein components (Garcia and Phillips, 2009), they are difficult to utilize for other non-feed applications (Garcia et al., 2006).

Hydrolysis has been used to improve the solubility and functionality of many bulk protein materials including wheat gluten (Wang et al., 2006), fish (Gildberg et al., 1989), animal hair (Coward-Kelly et al., 2006a), and soy protein isolate (Henn and Netto, 1998). Practical scale protein hydrolysis is usually achieved by catalysis with enzymes, acid, or alkali. In the present study we apply enzymatic and alkaline hydrolysis to three different animal protein meals (MBM, FM, and BM) in order to study the relative advantages of the two types of hydrolysis in transforming the meals into substances that are soluble, homogenous, and relatively free of non-protein substances.

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MATERIALS AND METHODS

ANIMAL PROTEIN MEALS AND HYDROLYSIS REAGENTS

Ruminant MBM and flash-dried cattle BM were obtained from Darling International (Irving, Tex.); hydrolyzed FM was obtained from Carolina By-Products (Winchester, Va.). The hydrolytic agents included Bell Mine hydrated lime, high calcium (Tannin Corp., Peabody, Mass.), Versazyme (BioResource International, Morrisville, N.C.), Alcalase 2.4L and Flavourzyme (Novozymes, Bagsvaerd, Denmark).

PROTEIN HYDROLYSATE PRODUCTION

The protein meals were partially defatted by brief extraction in a volume of hexane sufficient to permit easy

suspension of the meal and stirring of the mixture. This was followed by filtering through Whatman #1 filter paper. The extraction and filtering were repeated four additional times prior to hydrolysis. Hydrolysis was conducted in a thermostated reaction vessel with constant stirring adequate to prevent the settling of solids in the vessel. All reactions consisted of 9.1% (w/w) solid substrate in water. Alkaline hydrolysates were produced in 6-L batches, with 0.1-g CaOH/g substrate, at 85°C. Individual batches were hydrolyzed for 4, 8, or 16 h. Enzymatic hydrolysates were produced in 4-L batches at 50°C. Individual batches used one of three sets of conditions detailed in table 1. During enzymatic hydrolysis, pH was monitored continuously and maintained through the addition of 8M NaOH; use of concentrated NaOH allowed for pH control with negligible change in overall reaction volume.

Alkaline hydrolysis reactions were terminated by sparging with CO_2 until the pH dropped to 9, followed by neutralization with sulfuric acid. Enzymatic reactions were terminated by raising the reaction temperature to 90°C for 10 minutes. Residual solid material was removed by centrifugation using a Sorvall Instruments RC-3B centrifuge (Thermo Scientific, Asheville, N.C.) with an H6000A swinging bucket rotor, at 4200 rpm (5137 × g), refrigerated to 4°C, for 30 min. This was followed by filtration through a filtration capsule (Millipore, Billerica, Mass.) with 0.4-μm pores. The remaining hydrolysate was dehydrated using a Büchi B-191 Mini Spray Drier (Flawil, Switzerland).

Proximate Analysis

Moisture content determinations were performed by dehydrating 1-g samples at 70°C, under approximately 100-kPa (gauge) vacuum, for 24 h. Ash determination was conducted according to ASTM D 2617-96 (1996) which involves overnight incineration of samples in ceramic crucibles in a 600°C muffle furnace, using the dry samples from the moisture determination. Organic matter is reported as the difference between dry mass and ash mass.

Peptide Mass Analysis

Each sample was run through two different size exclusion columns using a Waters 2695 Separation Module and isocratic conditions. A Superdex Peptide 10/300 GL column (GE Healthcare, Piscataway, N.J.) was used to analyze the lower end of the molar mass range, and a BioSep-SEC-S 3000 column (Phenomenex, Torrance, Calif.) was used to analyze the higher end of the molar mass range. The eluting solvent for the Superdex column was 50-mM aqueous HCl; that for the BioSep column was 5-mM aqueous HCl containing 10% (v/v) acetonitrile. A Varian (Palo Alto, Calif.) 380-LC evaporative light scattering detector was used to quantify mass exiting the column.

Each column was calibrated against a wide range of standards. A calibration kit containing aprotinin, cytochrome C, carbonic anydrase, albumin, and bradykinin (Sigma, St. Louis, Mo.), as well as a separate kit containing throglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B_{12} (Biorad, Richmond, Calif.) were used in calibration. To extend the calibration to lower molar masses, these were supplemented with a Ser-Gly dipeptide and a pentapeptide (Peptide 6A), obtained from Bachem Americas (Torrance, Calif.). Data was transformed in manner that allows the combination of data from both columns, as well as plotting on a linear molar mass scale without loss of correlation between area under the curve and mass, using calculations described earlier (Garcia and Phillips, 2009).

Table 1. The combinations of substrate and hydrolysis conditions tested.

Hydrolysis Type	Conditions	Substrate		
		MBM	FM	BM
Enzymatic	Alcalase, 0.4 AU/g substrate, pH 8.5, 4 h	x	x	x
	Alcalase, 0.4 AU/g substrate, pH 8.5, 4 hours, then Flavourzyme, pH 7.0, 50 LAPU/g substrate, additional 4 h	x	x	x
	Versazyme, 8 mg/g substrate, pH 7.5, 8 h	x	x	x
Alkaline	Calcium hydroxide, saturated, 4 h	x	x	x
	Calcium hydroxide, saturated, 8 h	x	x	x
	Calcium hydroxide, saturated, 16 h	x	x	x

Amino Acid Analysis

Samples were hydrolyzed in triplicate using a PicoTag workstation (Waters Corp., Milford, Mass.) according to the manufacturer's directions. Hydrolyzed samples were filtered, dried under vacuum, and derivatized with AccQFluor reagent (Waters) following the manufacturer's directions. Chromatography was performed using procedures described as "mixture 1" by van Wandelen and Cohen (1997), with α-aminobutyric acid as an internal standard. Separate analyses were performed for cyst(e)ine, using the method described by Finley (1985) to quantitatively oxidize cysteine and cystine to cysteic acid prior to hydrolysis; these samples were then analyzed in the same manner as the other samples.

Data Presentation

The work described here resulted in large volumes of data; 18 different hydrolysis reactions were analyzed for multiple properties over multiple time points. To aid in comprehension and avoid long series of very similar figures, representative 'cross-sections' of the data have been selected for presentation.

Results and Discussion

Defatting

MBM and BM routinely contain about 12% (Garcia et al., 2006) and 1% (Dale and Batal, 2007; National Renderers Association, 2006) lipid, respectively, so the materials used in the present research were typical in this respect (table 2). FM is reported to normally contain about 4-6% lipid (Dale and Batal, 2007; National Renderers Association, 2006); the FM used here had a much higher fat content, almost 16%. Interviewing a renderer revealed that this elevated fat content could be explained by the addition of either whole birds or floatation grease from the rendering plant waste water treatment system to the feather cooker. He indicated that neither practice was uncommon (personal communication, David Kirstein, 18 June 2008).

Alkaline proteolysis is incompatible with the presence of lipid, because soaps are formed. In industrial practice, extraction to remove the lipid from the protein meals would likely be less aggressive than analytical extraction, and

Table 2. Lipid content of animal protein meals before and after hexane extraction.[a]

	% Lipid (dry basis)	
	As Received	Post Extraction
MBM	12.56 ±0.60	4.20 ±0.39
FM	15.84 ±0.83	7.24 ±0.16
BM	0.51 ±0.08	0.25 ±0.02

[a] Values are the average of three determinations ±1 standard deviation.

would allow some residual lipid to remain in the meals. The cursory fat extraction performed in the present research, i.e. several quick washings with hexane, was intended to model such an incomplete extraction. The results show that the extraction was even less complete than intended, removing only 50% to 65% of the lipid. Nevertheless, this residual fat did not create apparent problems.

Solubilization of Organic Matter

As discussed in an earlier paper (Garcia and Phillips, 2009), there are reasons to doubt the validity of both protein and nitrogen assays in determining the protein or peptide content of the protein meals and their hydrolysates. As will be shown later, the composition of the materials under consideration here range from free amino acids and dipeptides (ca. 110-220 Da) to entire proteins (ca. 100's kDa); protein assays cannot accommodate such extreme variation in a confounding factor. On the other hand, the various materials studied here are known to have very different amino acid compositions, but nitrogen-to-protein conversion factors are not available for these materials; application of the same factor to all the materials would produce misleading results. Consequently, our results use 'organic matter' as an imperfect proxy for protein content. This is reasonable considering these materials have negligible carbohydrate content, and the lipid content has been reduced.

Alkali and Versazyme hydrolysis both progressively solubilized the organic matter from each of the protein meals (fig. 1). The relatively slow and incomplete hydrolysis achieved by the alkaline hydrolysis was anticipated based on past studies with similar substrates, which indicate that alkaline proteolysis under atmospheric pressure proceeds slowly at 85°C (Coward-Kelly et al., 2006a; Coward-Kelly et al., 2006b; Kalambura et al., 2005), but equipment limitations did not allow us to use a higher temperature. By either hydrolysis method, BM was solubilized to a considerably lower extent than either MBM or FM; the reasons for this are not clear.

Molar Mass Distribution

It was hypothesized that hydrolysis would initially produce large soluble peptides, which would be progressively reduced in size as the hydrolysis reaction proceeded; this would allow the molar mass distribution in the solubilized portion to be controlled by stopping the reaction when the desired average molar mass was achieved. Analysis of samples taken from the reactions at various time points reveals, however, that the soluble portion comprises predominantly very small peptides throughout the reaction. Just 10 min into the reactions (fig. 2, solid traces), most of the solubilized mass consists of molecules with a mass of less than 1 kDa. Noting that these protein meals are rich in the smallest amino acid glycine (shown later), and that the rule-of-thumb for peptide molar mass is an average of 110 Da per amino acid, the main peaks in the chromatograms may represent free amino acids, and di- and tripeptides. The results after 8 h of hydrolysis (fig. 2, dashed traces) are similar, often showing a decreased proportion of peptides larger than 500 Da. Quantitative analysis of molar mass distribution (fig. 3) supports these observations. With either alkaline or enzymatic hydrolysis, the large majority of the peptides, represented by the number-average molar mass (M_n), have a small mass which does not change throughout the reaction. In the early stages of the reactions, however, there are minorities of soluble peptides which are much larger, which is reflected in the weight-average molar mass (M_w). M_w starts off high, especially in the alkaline reactions, and drifts down towards M_n as the large soluble peptides are repeatedly hydrolyzed into smaller pieces. Results from 8-h

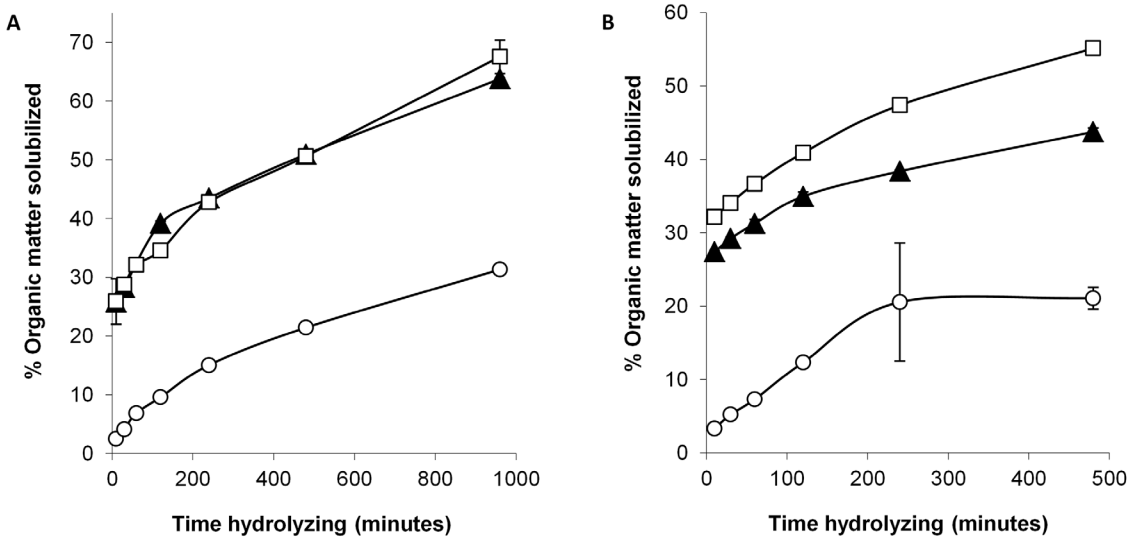

Figure 1. Change in solubility of organic matter throughout the course of the hydrolysis reactions. Results from (A) alkali and (B) Versazyme hydrolyzed MBM (▲), FM (□), and BM (○) are shown. Error bars are present for all points and represent ±1 standard deviation (s.d.) of two to three repetitions.

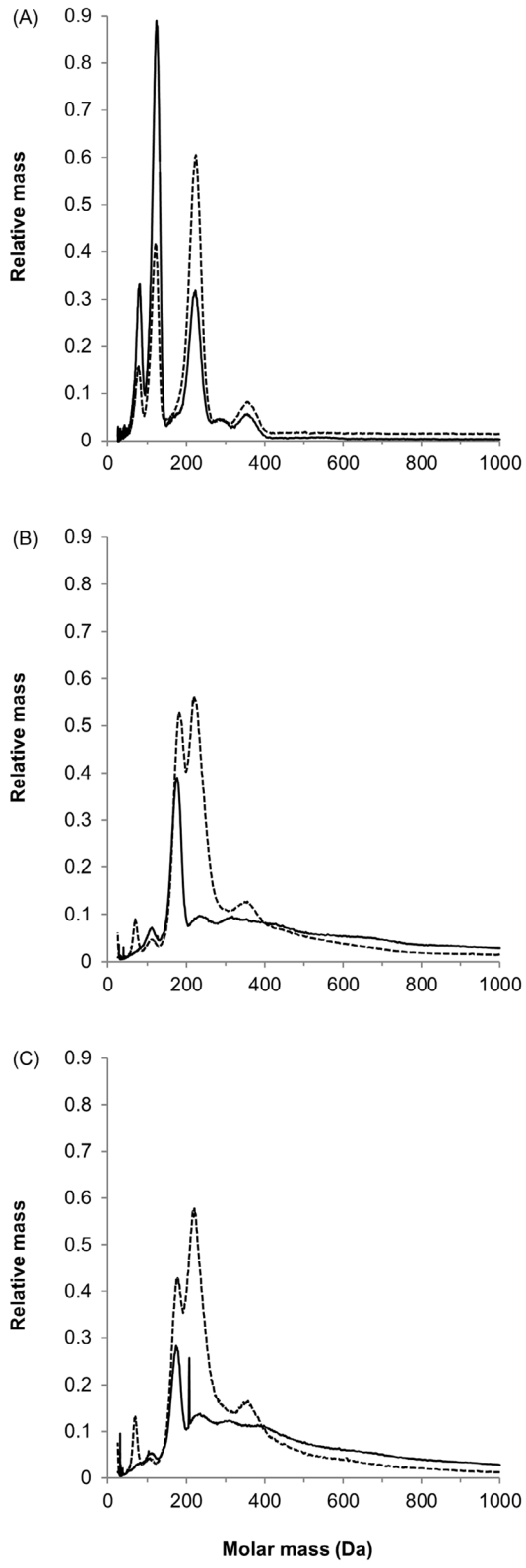

Figure 2. Representative transformed chromatograms from SEC analysis of hydrolysates. Data presented includes (A) alkali hydrolyzed MBM, (B) Alcalase and Flavourzyme hydrolyzed MBM, and (C) Alcalase and Flavourzyme hydrolyzed FM. Solid lines are from samples that have hydrolyzed for 10 min and dashes lines are from those that have hydrolyzed for 8 h.

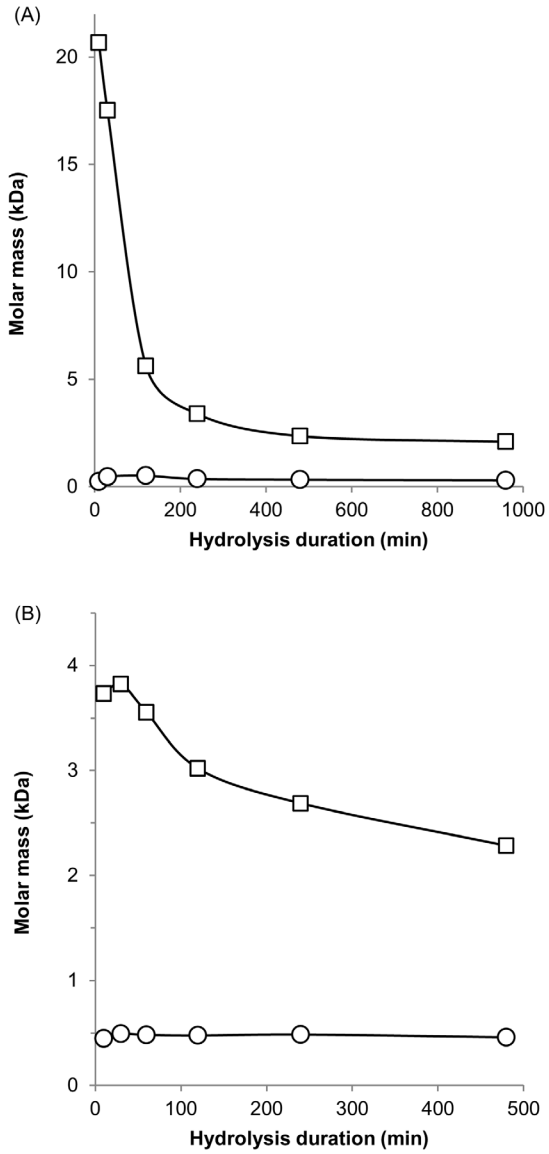

Figure 3. Number average molar mass (M_n; ○) and weight-average molar mass (M_w; □) of soluble peptides released from MBM throughout the course of (A) alkaline and (B) enzymatic hydrolysis reactions.

hydrolyses of each substrate by either method (table 3) show grossly similar results for all. The polydispersity indices (PDI) range from 3.5 to 7.4 indicating a fairly wide distribution of molar masses in each of the samples.

Amino Acid Composition

All batches of hydrolysate differed in amino acid composition compared to the protein meals they had been made

Table 3. The molar mass distribution of selected hydrolysates.[a]

	Alkaline			Enzymatic		
Substrate	M_n	M_w	PDI	M_n	M_w	PDI
MBM	320	2353	7.4	459	2284	5.0
FM	518	3691	7.1	459	1955	4.3
BM	676	2747	4.1	194	669	3.5

[a] Data labeled 'alkaline' is from batches hydrolyzed for 8 h; data labeled 'enzymatic' is from batches hydrolyzed with Versazyme.

Table 4. Relative amino acid composition of animal protein substrates and selected hydrolysate batches.[a]

Substrate	Meat & Bone Meal			Feather Meal			Blood Meal		
Hydrolysis Type	None	Alkaline	Enzymatic	None	Alkaline	Enzymatic	None	Alkaline	Enzymatic
Amino Acid	(parts per thousand)								
ala	68.3 ± 1.0	87.1 ± 0.7	73.4 ± 0.5	62.4 ± 0.1	76.4 ± 1.8	62.9 ± 0.4	76.9 ± 1.1	100.4 ± 1.2	78.4 ± 1.9
arg	73.9 ± 6.8	42.8 ± 0.1	67.4 ± 0.2	74.9 ± 0.0	46.6 ± 1.2	71.3 ± 3.7	41.3 ± 0.1	24.5 ± 0.2	35.7 ± 0.3
asx	78.6 ± 1.1	71.8 ± 0.1	73.3 ± 0.6	82.8 ± 0.4	80.2 ± 4.0	79.1 ± 0.6	100.4 ± 0.7	121.2 ± 1.4	103.2 ± 1.5
cys	8.3 ± 3.4	< dl	2.4 ± 1.0	4.7 ± 2.2	1.9	5.2 ± 0.2	5.4 ± 1.9	1.5	3.5 ± 0.5
glx	141.6 ± 2.1	141.2 ± 0.5	144.4 ± 0.7	141.3 ± 0.7	144.1 ± 6.0	144.1 ± 1.1	89.5 ± 0.5	92.1 ± 0.8	93.4 ± 1.3
gly	107.7 ± 2.1	168.6 ± 0.4	135.8 ± 0.3	85.3 ± 0.4	141.0 ± 4.7	102.9 ± 0.8	37.8 ± 0.2	74.5 ± 0.8	39.7 ± 0.3
his	25.5 ± 0.5	24.8 ± 0.6	26.1 ± 0.5	26.5 ± 0.2	23.4 ± 0.3	22.6 ± 0.2	63.5 ± 0.3	63.7 ± 0.8	63.1 ± 0.8
hyl	2.7 ± 0.2	7.2 ± 0.6	7.2 ± 0.7	2.3 ± 0.1	5.6 ± 0.2	4.9 ± 0.2	1.1 ± 0.3	1.5 ± 0.2	2.9 ± 1.1
hyp	21.5 ± 0.4	72.2 ± 0.4	65.1 ± 0.4	13.6 ± 0.1	47.9 ± 1.1	39.4 ± 0.7	0.4 ± 0.0	2.8 ± 0.0	1.5 ± 0.0
ile	36.8 ± 1.0	25.0 ± 0.5	27.6 ± 0.3	44.8 ± 0.3	35.8 ± 0.2	36.9 ± 0.4	5.8 ± 0.0	6.9 ± 0.6	7.1 ± 0.1
lal	< dl	3.3 ± 0.5	< dl	< dl	10.3 ± 0.0	< dl	< dl	25.7 ± 1.0	< dl
lan	< dl	< dl	< dl	< dl	< dl	13.4 ± 2.8	< dl	< dl	< dl
leu	72.0 ± 1.0	67.8 ± 1.2	57.9 ± 0.3	78.5 ± 0.7	76.6 ± 0.1	65.2 ± 1.1	129.0 ± 0.4	109.3 ± 1.4	125.7 ± 1.1
lys	51.4 ± 0.4	35.4 ± 0.3	41.7 ± 0.4	64.1 ± 0.7	40.1 ± 1.8	57.4 ± 0.3	96.5 ± 1.1	88.6 ± 0.3	95.6 ± 0.4
met	24.6 ± 5.8	12.8 ± 0.3	13.3 ± 2.2	15.8 ± 3.1	15.1 ± 1.6	18.9 ± 1.1	14.0 ± 3.2	26.0 ± 1.3	18.6 ± 2.5
NH_3	17.9 ± 2.4	14.6 ± 0.9	16.1 ± 1.7	18.4 ± 0.5	15.8 ± 1.0	15.0 ± 0.5	13.3 ± 0.5	11.4 ± 1.5	12.9 ± 1.0
phe	40.1 ± 0.6	31.2 ± 0.6	32.5 ± 0.4	44.6 ± 0.3	38.9 ± 0.7	35.8 ± 0.3	78.2 ± 0.3	60.9 ± 0.7	70.5 ± 0.5
pro	80.0 ± 1.7	110.7 ± 3.6	92.4 ± 2.3	66.6 ± 0.2	93.9 ± 3.3	72.5 ± 1.4	36.1 ± 0.5	41.2 ± 1.7	40.0 ± 2.7
ser	34.2 ± 0.8	14.0 ± 0.4	32.0 ± 0.4	46.3 ± 0.5	19.5 ± 0.5	44.7 ± 0.8	46.3 ± 0.5	20.0 ± 0.5	48.0 ± 0.8
thr	30.6 ± 0.7	9.4 ± 0.2	26.8 ± 0.2	37.0 ± 0.3	11.0 ± 0.4	35.4 ± 0.1	43.9 ± 0.4	10.6 ± 0.2	46.9 ± 0.5
tyr	34.2 ± 0.8	20.8 ± 0.1	23.6 ± 0.6	36.6 ± 0.4	29.8 ± 1.0	27.7 ± 0.3	31.9 ± 0.3	36.4 ± 0.4	28.4 ± 0.5
val	50.1 ± 0.8	39.2 ± 0.7	40.9 ± 0.7	53.6 ± 0.6	46.9 ± 0.3	44.9 ± 0.3	88.6 ± 0.9	81.7 ± 1.1	84.9 ± 0.9

[a] Batches labeled 'alkaline' are those that were hydrolyzed for 16 h; batches labeled 'enzymatic' are those treated with Versazyme. Values are the average of 2 to 3 repetitions +1 standard deviation. "Lal" is the crosslinked amino acid lysinoalanine; "lan" is the crosslinked amino acid lanthionine; "glx" is the sum of glu and gln; asx is the sum of asp and asn. "<dl" means that the concentration of that component was below the detection limit for the analytical method used. Values without a standard deviation are the result of analyses in which only one repetition produced a value greater than the detection limit.

from (table 4). Such differences may be the result of at leasttwo different mechanisms; either certain amino acids are destroyed or created during the hydrolysis reaction, or the different protein components of the meals are not solubilizing at a uniform rate, resulting in a solubilized portion with a composition that is not representative of the original meal.

In all alkaline hydrolysis experiments, the relative concentrations of arginine, serine and threonine decreased progressively with increasing reaction time (fig. 4a), suggesting that these amino acids are labile under the conditions used in the reaction. This finding is consistent with the results of an earlier study involving the alkali digestion of cattle hair (Coward-Kelly et al., 2006a). Enzymatic hydrolysis had little effect on the relative concentrations of these same amino acids (fig. 4b). Additionally, the decomposition of some amino acids in alkaline conditions yields different amino acids; serine and threonine both decompose to yield glycine and alanine, and cysteine and cystine yield alanine, among other decomposition products (Hill, 1965). This is consistent with the increase in alanine and glycine observed in alkali-hydrolyzed samples.

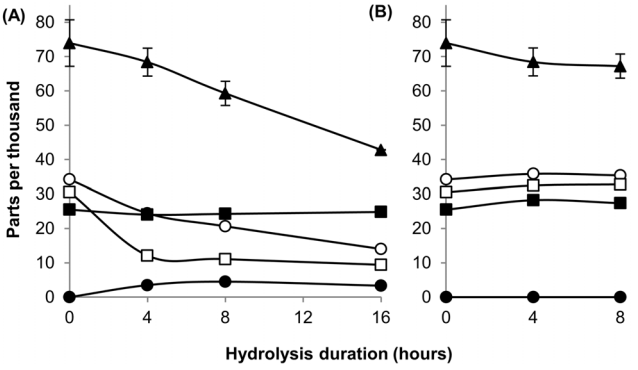

Figure 4. The progressive change in the relative concentration of some amino acids during hydrolysis of MBM. Data are from (A) alkali and (B) Alcalase and Flavourzyme hydrolyzed batches. Data presented includes values for arginine (▲), serine (○), threonine (□), lysinoalanine (●); histidine (■), the concentration of which does not progressively change, is included for contrast. Each data point represents the average of two to three measurements, and error bars representing one standard deviation are present for each point.

Alkali treatment of protein is known to result in the formation of some unusual cross-linked amino acids including lysinoalanine and lanthionine (Friedman, 1999). Lysinoalanine was absent from all samples of the protein meals as well as all samples of enzymatic hydrolysates. Lysinoalanine was, however, present in every alkali-hydrolyzed sample tested. The highest relative concentrations of lysinoalanine were found in alkali-hydrolyzed BM; this may be the result of BM's relatively high concentration of lysine, a precursor to lysinoalanine. Lanthionine was absent from all

rendered proteins; it was detected in most alkali- and enzymatic-hydrolyzed feather meal samples and a one type of enzymatically-hydrolyzed MBM. The mechanism for lanthionine formation in enzymatically-hydrolyzed batches is unclear.

All alkali- and Versazyme-hydrolyzed samples had increased concentrations of proline, hydroxyproline, glycine, and hydroxylysine; Alcalase- and Flavourzyme-hydrolyzed samples were not altered in this manner. Since collagen is the only significant biological source of hydroxyproline and hydroxylysine, and collagen is unusually rich in glycine and proline, we hypothesize that the increased concentration of these amino acids in the hydrolysates is due to preferential hydrolysis of collagen in the protein meals. Although neither blood nor feathers have significant collagen content, discussions with renderers regarding industrial practice revealed that in many cases neither blood meal nor feather meal is made exclusively from blood or feathers, respectively. As mentioned earlier, some whole birds may be included in feather meal production, and blood meal may contain some MBM, due to the use of shared conveyance, processing and storage equipment (personal communication, Chad Kuzel, 28 June 2007).

Proximate Analysis of Protein Hydrolysate

One goal of the present project was to produce hydrolysate products that are low in ash; success along these lines was only modest considering that many hydrolysates had ash contents similar to or greater than the animal protein substrates they were made from (table 5).

The high ash content of unhydrolyzed MBM is known to be mainly due to the presence of the bone particles (Garcia and Phillips, 2009); muscle tissue contains only about 4% ash on a dry basis (Field, 2005). Poultry feathers themselves are about 6% ash on a dry basis (Dalev, 1994), but as discussed earlier, the FM used in the present study likely included whole birds, and consequently, bones. The ash portion of bone is predominantly hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Narasaraju and Phebe, 1996) which has very low solubility ($K_{SP} = 2.91 \times 10^{-58}$) under the conditions used in either the enzymatic or alkaline hydrolyses (Bell et al., 1978), and very little insoluble bone mineral is likely to have remained in the hydrolysate product after the centrifugation and filtering processes.

Table 5. Proximate analysis of animal protein substrates and representative hydrolysate batches.[a]

Substrate	Treatment	% Moisture	% Organic Matter (d.b.)	% Ash (d.b)
MBM	Unhydrolyzed	3.3 ±<0.1	74.3 ±0.7	25.7 ±0.7
	Alkaline	2.9 ±0.2	77.2 ±0.5	22.8 ±0.5
	Enzymatic	1.7 ±0.4	85.1 ±0.5	14.9 ±0.5
FM	Unhydrolyzed	4.2 ±0.2	86.3 ±0.6	13.7 ±0.6
	Alkaline	2.5 ±0.2	79.5 ±0.1	20.5 ±0.1
	Enzymatic	0.7 ±0.3	89.0 ±< 0.1	11.0 ±< 0.1
BM	Unhydrolyzed	10.3 ±0.1	97.9 ±0.1	2.1 ±0.7
	Alkaline	2.2 ±0.3	85.3 ±0.2	14.7 ±0.2
	Enzymatic	0.3 ±0.1	91.5 ±0.1	8.5 ±0.1

[a] Batches labeled 'alkaline' are those that were hydrolyzed for 16 h; batches labeled 'enzymatic' are those treated with Versazyme. Values reported are the average of three repetitions ±1 standard deviation.

Rather than being carried over from the substrate, ash in the hydrolysates originated with reagents used in the reaction. The ash contents of the alkali-hydrolyzed batches ranged from 14.8% to 27.2% (d.b.). Aspects of the alkaline hydrolysis system were selected specifically to minimize ash. Neutralization of a calcium hydroxide solution with carbon dioxide yields poorly soluble calcium carbonate ($K_{SP} = 8.7 \times 10^{-9}$) and soluble calcium bicarbonate ($K_{SP} = 1.08$). Coward-Kelly et al. reported that maximum precipitation of calcium salts from a saturated calcium hydroxide solution resulted from acidification of the solution with CO_2 to pH9; below pH9 presumably the proportion of the bicarbonate to carbonate salt increases, resulting in greater calcium solubility (Coward-Kelly et al., 2006b). Neutralization of a calcium hydroxide solution with sulfuric acid yields calcium sulfate (gypsum; [$K_{SP} = 6.1 \times 10^{-5}$]), which is more soluble than calcium carbonate, but still poorly soluble (Criswell et al., 1964). Our practice of sparging the reaction mixture with CO_2 until the pH dropped to 9, followed by adding sulfuric acid until the reaction mixture was neutralized, was intended to minimize the residual salt in the solution. Clearly, the success of this strategy was limited. Further experimentation, reported elsewhere (Garcia et al., 2010), confirmed that approximately half of residual ash in solution consisted of calcium salts. Possibly, calcium that precipitated as calcium carbonate re-solubilized as calcium bicarbonate when the solution was neutralized.

The ash content of the enzymatic batches was lower, ranging from 8.5% to 15.1% (d.b.) All batches employed an alkaline protease and required significant additions of sodium hydroxide to maintain the optimum pH as the reactions proceeded. Nevertheless, enzyme hydrolyzed batches made from MBM or FM had lower ash content than the starting material. The ash content of BM hydrolysate was greater than the starting material; it can be speculated that the hemoglobin in BM was preferentially hydrolyzed, relative to BM components that remained insoluble, and that heme iron contributed disproportionately to the ash content of the hydrolysate.

Conclusions

Insoluble, non-homogeneous rendered meals can be processed into soluble, homogenous hydrolysates through either enzyme- or alkali-catalyzed lysis. Such processing increases the utility of the protein in the rendered meal. The hydrolysates produced through the present research have been tested as bio-based flocculants; some, particularly those produced through alkaline hydrolysis were found to be effective (Piazza and Garcia, 2010). The hydrolysates have also been tested as feedstocks for industrial fermentations, substituting for expensive ingredients such as yeast extract or casamino acids. They were found to have favorable performance characteristics for this application (Garcia et al., 2010), and support the growth of the bacterium *E. coli*, the fungus *Pythium irregular*, and the alga *Schizochytrium limacinum*, in valuable-product-producing fermentation systems (manuscripts in preparation). The unhydrolyzed rendered meals are not effective in these applications. The observed presence of small amounts of cross-linked amino acids in the hydrolysates probably has no effect on the 'functionality' of the hydrolysates, but it may have a negative

impact on the nutritive value of the hydrolysates for microorganisms. The remaining challenges include achievement of a more complete conversion of the raw material, development of techniques to control the size of peptides produced, and improvement of the process economics.

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